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Proteolytic Enzymes...

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Nucleic Acid Sequences Coding for Proteolytic Enzymes in the Form of Specific Proteases, Corresponding Polypeptides and Use Thereof

BACKGROUND OF THE INVENTION.

1. Field of the Invention.

The invention relates to nucleic acid sequences coding for proteolytic enzymes in the form of specific proteases, to corresponding polypeptides and to the use thereof.

2. The Prior Art.

Proteases (also known as proteinases) are protein-splitting enzymes (proteolytic hydrolases) distinguished by their effective mechanisms. Proteins are typical folded chains of polypeptides. For that reason, proteases are also called peptidases and are distinguished between endo- and exo-peptidases. Endo-peptidases split the peptide chains in their interior and thus generate smaller fragments; endo-peptidases can split off the terminal amino acids. Proteolytically active enzymes are present everywhere in biological organisms and satisfy purposes specific to a given type. Thus, they may, on the one hand, be function-relatedly used for therapeutic purposes or they may serve in technical purification purposes against proteinaceous contaminations. The families of calpain-proteases (CANP) and metalloproteases (MP) are important representatives of proteases and the subject of intensive research.

Calpain-7-Protease

To date, about 16 different CANP are known. The CAPN most widely researched consist of large 80kDa (kilo Dalton) sub-unit and a small 30kDa sub-unit. The large sub-unit consist of four and the small sub-unit consist of two functional domains. The IV domain of the large and the VI domain of the small sub-unit are formed regularly over their folding shapes ("EF-hands") as calcium-bonding domain. It is assumed that the proteolytic activity of this CAPN is released only by bonded calcium atoms. By contrast, the later CAPN (5,6,7,10,13; see, for instance, Publication I) have no such calcium-bonding structures in domain IV of the large sub-unit. All members of the CAPN family are always tissue-specifically structured and in this fashion satisfy the most variegated tasks.

Often, CAPN assume key positions in metabolistic paths and participate in pathogenesis. For instance, the CAPN 1 and 2 are components of the reaction cascade of apoptosis ("programmed cell death") and are thus participating in the course of Alzheimer's disease. CAPN may also act upon carcinoses. In this connection, breast cancer and intestinal cancer, for instance, are triggered by an increase of the cellular concentration of the p53 marker protein. The increase in the concentration is caused by inhibiting the participating CAPN which, in healthy cells, normally keep the p53-concentration very low (see Publication I: "The calpain family and human disease", Yuanhui Huang and Kevin K. W. Wang in Trends in Molecular Medicine, Vol. 7, No. 8, August 2001, pp. 355-362).

Calpains play a special role in the cell migration in the extra-cellular matrix. Cells migrate by dissolving at their rear end and by synthesizing at their front end. Calpains participate in this migration by proteolytic splitting of protein complexes of the support apparatus at the end of the cell (see Publication II: "V-SRC's hold over actin and cell adhesions", Frame, Fincham, Carragher, Eyke in Nature Reviews, Molecular Cell Biology, Vol. 3, April 2002, pp. 233-245).

The described findings are based on experiments with invertebrates, mammals and humans. CAPN in plants and fungi have hitherto been identified only be genome- and EST projects. In plants, a pathogenic defense effect and, in fungi, adaptation to alkaline living conditions are assumed. CAPN is not known in the entire class of diatomacae.

Enzymes from the calpain family have been disclosed in the patent literature, for instance, in EP 1,214,427, CA 2,321,270 and DE 100 31 932. Those publications constitute the technical and scientific background of the present invention.

Zinc metalloprotease

Metalloproteases (MP) are regularly contained, among others, in members of the so-called ADAM family (a disintegrin and metalloprotease) of the trans-membrane proteins which consist of a desintegrin and a MP domain and which develop cell adhesion and protease activity in connection with the events during fertilization, the development of nerve tissue and during inflammation reactions (see Publication III: "Autotrope Singnaltransduktion durch membranstaendigen Tumor-Nekrose-Faktor TNF", Doctoral Dissertation University of Stuttgart, Germany, E. Haas, 1999, pp. 6-8).

Furthermore, metalloproteases play an important role in the renin-angiotensin-aldosterone system (RAS). The aspartyl protease renin cleaves angiotensinogen into angiotensin I. The angiotensin converting enzyme (ACE), which thereafter cleaves angiotensin I into angiotensin II is a zinc metalloprotease (ZnMP). Angiotensin II has a vascoconstrictive and, hence, blood pressure raising effect and promotes the release of aldosterone in the cortex of the suprarenal gland which, in turn, leads to cardiac hypertrophy. Thus, ACE is an important substance for regulating the circulatory function and requires suppression in certain cardiovascular diseases (ACE inhibitor) (see Publication IV: "Cardiovascular Physiology Concepts, University of Ohio,

R. E. Klabunde, 4 June 2002 from http://www.oucom.ohiou.edu/CVPhysiology/BPO15.htm (Status 20 August 2002).

Moreover, as collagenases metalloproteases contribute to the decomposition of sustentacular tissue in the body. Collagens are structural proteins and as the protective main component of intercellular support substance they protect 25% of the body tissue against enzymatic attacks. By the intercalation of mineral crystals, collagens may develop into hard bone and tooth substances. In the life cycle of collagens, collagenases are of importance in connection with chemo-nucleolysis, i.e. a method of treating intervertebral disk lesions by dissolving the nucleus of the intervertebral disk (nucleus pulposus).

Enzymes from the metalloprotease family have been disclosed in the patent literature, e.g. WO9964610 (zinc metalloprotease), US 2002068055 (metallo endopeptidase) and US 5,750,391 (metallo endopeptidase). The publications constitute technological and scientific background in respect of the present invention.

From the publication "Zahllose Geheimnisse der Natur" by K. Eske (see BioLOG, 3rd edition, February 2000, pp. 2-3), dowloadable from http://www.bioregio.org/BiolOG-3.pdf - mind the upper and lower case letters when downloading from the Internet ! - status 1 September 2002) it is known to isolate coldness-adapted enzymes from bacteria occurring in deep sea regions. In addition to the advantage of coldness-adapted enzymes not requiring increased temperatures for their expression, the potential of occurrence of the corresponding organisms from deep sea regions is large in view of the fact that 80% of the water covering the earth is of a temperature of below 5 °C. The temperature of the maximum activity of these organisms and their functional components is significantly below that of other organisms from temperate and tropical areas. Compared to conventional production

processes with organism which are not coldness-adapted, the use of coldness-adapted organisms, including those of genetically modified form, allows production at low temperatures and is significantly more economical than at their usual activity temperatures.

OBJECT OF THE INVENTION.

With the background of these significant findings, it is an object of the present invention for purposes of an economical production of the proteases in accordance with the invention to find a coldness-adapted organism with nucleic acid sequences coding for coldness-adapted enzymes in the form of specific proteases at low temperatures (around 0 °C).

SUMMARY OF THE INVENTION.

The object is accomplished in the manner defined in claim 1. Two different nucleic acid sequences from the coldness-adapted diatom *fragilariopsis cylindrus* are being claimed, one of which codes for a calpain-7-protease and the other one for a zinc metalloprotease. Advantageous improvements and applications which also relate to the polypeptides corresponding to the claimed nucleic acid sequences, have been defined in the subclaims and a the auxiliary claims.

The solution in accordance with the invention optimally satisfies the demands placed on the invention. Firstly, the organism provided with the claimed nucleic acid sequence is provided with specific proteases in the form of a calpain-7-protease enzyme as well as of a zinc metalloprotease enzyme, and, secondly, the organisms comes from the antarctic sea so that these enzymes are coldness-adapted so that no heating is required for their activity. The knowledge of such genes containing the claimed nucleic acid sequences is of elementary importance where large quantities of such nucleic acid sequences must be made available at low energy consumption. The

microbial synthesis of these substances leads to high yields at low temperatures with the particular rapidly growing and coldness-adapted *frangilariopsis cylindrus* diatom.

DESCRIPTION OF PREFERRED EMBODIMENTS.

Sequence analyses which have been performed have confirmed that the nucleic acid sequences are coding for a calpain-7-protease and for a zinc metalloprotease. It has become possible, by means of current developed methods of automatically sequencing nucleic acid sequence sections, within a reasonable time specifically to isolate from promising organisms genes of the desired properties. Large public data bases containing sequences of defined and known functions serve for more rapidly verifying the results of the arduous search. A number of operational steps known *per se* is used to provide from the specific diatom the basic material prepared for the sequencing:

1) Isolation and cultivation of the organism *frangilariopsis cylindrus*

Isolation: The diatom *frangilariopsis cylindrus* was isolated from sea ice during an antarctic voyage onboard the German research icebreaker to the Weddell Sea. Defining the species was accomplished in a simple fashion by typing the structure of its shell (see Publication VI by Medlin & Priddle: "Polar Marine Diatoms", 2nd Edition, British Antarctic Survey, Cambridge, 1990, pp. 182, 192).

Cultivation: The diatom was maintained at 0 °C in a nutrient salt enriched medium 2 x f/2 at 10 µmol photons $m^{-2}s^{-1}$ at 24h light (see Publication VII by Guillard & Ryther: "Studies of marine plankton diatoms, I. Cyclotella nana (Husted) and Detonula confervacea (Cleve)", 1962, Can. J. Microbiol. 8, pp. 229:239). Halfway through their exponential growth phase the algae were cooled to -2 °C for an increased expression of the genes

responsible for the coldness-adjustment of the species. This corresponds to the ice point of sea water. After five days the messenger RNA (mRNA) were isolated from the algae. All of the mRNA represented straight active genes, even those responsible for the coldness-adjustment.

2.) Isolation of the MRNA

The entire RNA was isolated with the RNAeasy Plant Mini Kit (Qiagen Company). About 800 ng of RNA for the cDNA synthesis could be isolated from about 100 µg of RNA with the Oligotex mRNA Midi Kit (Qiagen Company).

3) Production and Screening of a cDNA Bank.

The cDNA bank was produced on the basis of the mRNA with the SMART cDNA Library Construction Kit (Ciontech Company).

A) For this purpose, the first cDNA strain was synthesized from the mRNA with the aid of oligonucleotides and CDS III/3' primers.

B) Thereafter, the double strain synthesis was carried out with the aid of the LD-PCR (long distance polymerase chain reaction) in an Eppendorf thermocycler following the following program:

C)

1. 5 min of denaturizing at 95 °C followed by 20 cycles of 6 min at 68 °C and 2 min at 95 °C.
2. After a Sfil digestion (with restriction enzyme from streptomyces fimbriatus) of the cDNA it was fractionated according to size in CHROMA Spin-400 columns so that only cDNA's of a length > 400bp (base pairs) were used for cloning;
3. The CDNA were ligated over night at 16 °C in TriplEX2 vectors which could be received by λ-phages. The titre of the cDNA bank was about 2.7×10^9 pfu/ml (plaque forming units / ml).
4. Blue-white screening with IPTG (isopropyl-b-D-thiogalactoside)

and X-Gal (X-galactoside) displayed a recombination efficiency of 70%.

4. Sequence Analysis

Positive phage plaques were sequenced by the Qiagen Sequencing Service from their 5'-end with λ-primers. The sequences were examined for their homologies to existing sequences (BLAST Protocol of 14 December 2001, calpain-7-protease; BLAST Protocol of 24 April 2002; zinc metalloprotease). For the calpain-7-protease, a homologous sequence could be found from about 400 sequences; for the zinc-metallo protease at least one homolous sequence could be found.

Figure 1 depicts the result of a phylogenetic analysis for the calpain-7-protease from the *fragilariopsis cylindrus* diatom. With a significant bootstrap support (generally known mathematical method) of 99% it is grouping with other calpain-7-proteases. Figure 2 depicts the result of a phylogenetic analysis of zinc metalloprotease from *fragilariopsis cylindrus*. With significant bootstrap support of 99% it is also grouping with other zinc metalloproteases.

Therefore, with 99% certainty, the coldness-adapted enzymes coded from the claimed nucleic acid sequence in accordance with the invention from the *frangilariopsis cylindrus* diatom are also a calpain-7-protease and zinc metalloprotease, respectively.